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Table of Contents

INTRODUCTION

This annual report covers the period September 1st , 2000 through August 31st, 2001. The tasks outlined in the Statement of Work that are applicable to this funding period are Tasks 1 through 6. Below, I have included brief summaries of the work accomplished with data, where applicable. We are slightly behind schedule with regard to our timeline due to two reasons: first, the cloning of the constructs was slightly more time consuming because it entailed the ligation of blunt-ended constructs. The second reason for being behind schedule is the problem we encountered with Task 4. This is explained below. At this point in time, the work will form a basis for ongoing research activities but is short of a full publication. However, we are moving forward constructively with our experiments.

RESEARCH ACCOMPLISHMENTS

Task 1: Subclone the p90^{rsk} alleles into a mammalian expression vector.
Proposed Time Line: Year 1/Month 1
Actual Time: Year 1/Month 2.5

P90^{rsk} has been cloned into a retrovirus which was used to infect cells with extremely high efficiency. Retroviruses are enveloped and have RNA genomes that are typically approximately10 kb in size . Upon infection, the RNA genome is converted to double stranded DNA by the virally-encoded reverse transcriptase. The proviral cDNA integrates into the genome of the host through the activity of the virally-encoded integrase. Retroviral vectors that have all of the retrovirus genes removed and replaced by the gene of interest (in this case) are efficiently packaged either in cell lines that stably express the gag, pol, and env proteins or through transient transfection with plasmids that encode the gag, pol, and env proteins and the provirus of the vector. Env is a viral transmembrane protein that binds to the viral receptor on the host cell to initiate cellular uptake of the virus.

The genes were cloned into an HIV vector that encodes resistance to the antibiotic G418. A parallel construct with empty vector (*i.e.* p90rsk allele removed) was made as a control. Using transient transfection of 293T cells, virus vectors can be generated. The vectors were pseudotyped using VSV-G and concentrated by centrifugation. The concentrated virus was titered using a colony forming assay in 293 cells and then used to transduce various cell lines including an immortalized human mammary epithelial cell line obtained from Clontech (hTertMam) and the MCF-7 breast carcinoma cell line. The cells were put under selection with G418 and cloned. Expression of p90rsk was examined and cellular growth characteristics were also examined.

Task 2: Determine their expression upon transfection by Western analysis.
Proposed Time Line: Year 1/Month 2-3
Actual Time: Year 1/Month 2.5 - 4.5

Two cell lines were chosen for initial studies. The first was a primary breast epithelial cell line immortalized with human telomerase (hTert). This cell line has elements of the p16INK cell cycle regulatory pathway inactivated as well, but has characteristics that are very consistent with untransformed cells. However, it has the advantage of being immortal due to the expression of telomerase. The second cell line chosen for study was the MCF-7 cell line obtained from Dr. Kim Leslie's laboratory at the University of Colorado Health Sciences Center.

Figure 1 depicts the results of our transduction efforts. Basically, single cell clones of transduced cells were cultured in 96 well plates until a sufficient number of cells were present for culturing. The cultures were grown in two T25 flasks until confluence; one set of flasks was used for freezing cells while the other was used for analysis of protein by Western blotting. Both hTertMam and MCF-7 cell lines expressing kinase dead (KD), wild-type

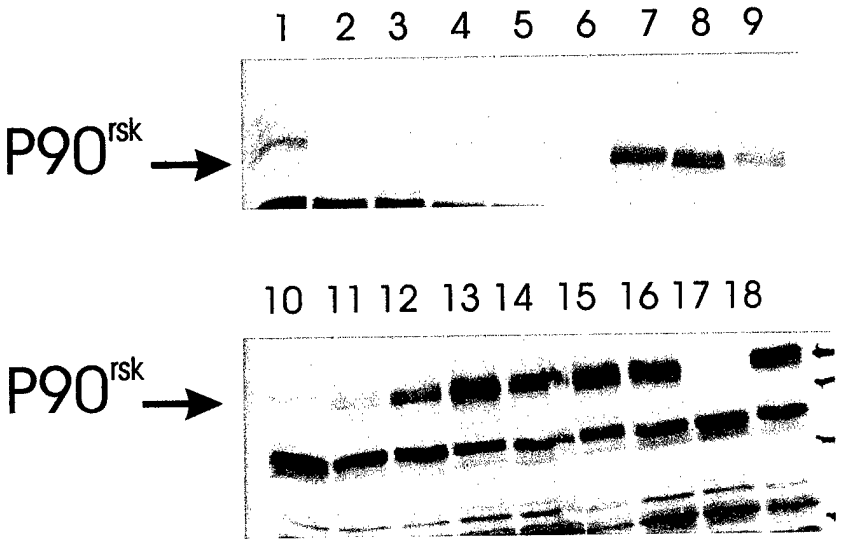

Figure 1: Expression of HA-tagged p90rsk alleles in HtertMam and MCF-7 cell lines. Western blot analysis using anti-HA of a panel of cell clones derived from virus-transduction. Virus carried the vector, kinase-dead, wild-type, or constitutively active constructs of p90rsk. Baculovirus produced HA-tagged p90rsk (lane 1); untransduced cell lines (2,3); vector transduced (4,5); kinase-dead (6-10); wild-type (11-14); constitutively active (15-18). The telomerase expressing human mammary cell line (hTertMam) cells were analyzed in lanes 2,4,6-8,11,12,15 and 16. MCF-7 cells were analyzed in lanes 3,9,10,13,14,17, and 18. Many more clones were obtained and archived but not tested. Not all clones tested were positive. For example, lanes 6, 10 & 17 did not appear to be expressing p90rsk.

(WT) and constitutively active (CA) were obtained as shown and described (Figure 1). The cell lines derived will be referred to as shown in Table 1.

TABLE 1	P90^{RSK} EXPRESSING CLONES DERIVED FROM HTERTMAM AND MCF-7						
	Vector	Kinase-Dead		Wild-Type		Constitutively Active	
HTertMam	HMV-4	HMKD-7	HMKD-8	HMWT-12	–	HMCA-15	HMCA-16
MCF-7	MCV-5	MCKD-9	–	MCWT-13	MCWT-14	MCCA-18	–

Task 3: Determine the kinase activity of the expressed p90^{rsk} alleles by immunoprecipitation/kinase assays using the hemagluttinin-tag (HA) epitope directed antibody.
Proposed Time Line: Year 1/Month 4
Actual Time: Year 1/Month 4.5 - 6

Extracts of cells positive for expression of p90^{rsk} were used to determine whether protein kinase activity could be detected from the different alleles of p90^{rsk}. The methodology used was to immunoprecipitate HA-tagged p90^{rsk} from cell extracts and assay for phosphorylation of bacterially produced IκBα. IκBα was chosen because our lab had previously identified this protein as being quantitatively phosphorylated by p90^{rsk} *in vitro* (Ghoda, 1997). We have been able to detect kinase activity associated with the immunoprecipitates for WT and CA alleles (Figure 2). The immunoprecipitate for the KD allele (lanes 9 & 10) were exposed for 18 hrs and reveals some phosphorylation of IκBα. We suspect this may be due to wild-type p90rsk coprecipitating with the HA-tagged protein. See discussion below for Task 4. P90^{rsk} most likely engages in autophosphorylation as the bands in the WT and CA lanes are more heavily phosphorylated than the bands in the KD lanes. The ^{32} P-labelled bands migrating at the molecular weight of p90^{rsk} in the KD lanes may or may not be p90rsk as the vector lanes also contain a band at about the same molecular weight that are likely to be background bands.

Task 4: Determine the effects of transfect p90^{rsk1} on endogenous p90^{rsk1}.
Proposed Time Line : Year 1/Month 5
Actual Time: Year 1/Month 7-8

Extracts of cells positive for expression of p90^{rsk} were used to determine whether expression of KD or CA (in particular) would influence the activation state of the endogenous p90^{rsk}. The methodology employed was a modification of the method used in Task 3, namely, following immunoprecipitation with anti-HA antibody which would presumably precipitate all tagged p90^{rsk}, anti-p90^{rsk} antibodies were used to precipitate the endogenous untagged protein. This should have been straightforward, but we were never able to recover much kinase activity corresponding to endogenous p90^{rsk} following this procedure. The activity indeed appeared to be only 20% of the parental cell lines. If the anti-p90^{rsk} antibodies were used first, we could detect precipitated kinase activity. If the anti-p90^{rsk} precipitates are probed with anti-HA antibody in an IP-western assay, HA-tagged p90^{rsk} could be detected. Likewise, if HA-tagged immunoprecipitate is probed with anti-p90^{rsk} plenty of p90^{rsk} protein can be detected (data not shown). On our gel system, it was not possible to tell if the immunoprecipitated band represented both tagged and untagged protein. We are presently modifying the gel conditions to allow the separation of the tagged proteins from the untagged. We are also trying different batches of antibodies directed towards p90^{rsk}.

One possible explanation is the possibility that the HA-tagged p90^{rsk} is forming complexes with the endogenous protein and co-immunoprecipitating the protein. This could explain why p90^{rsk} is evidently present but not

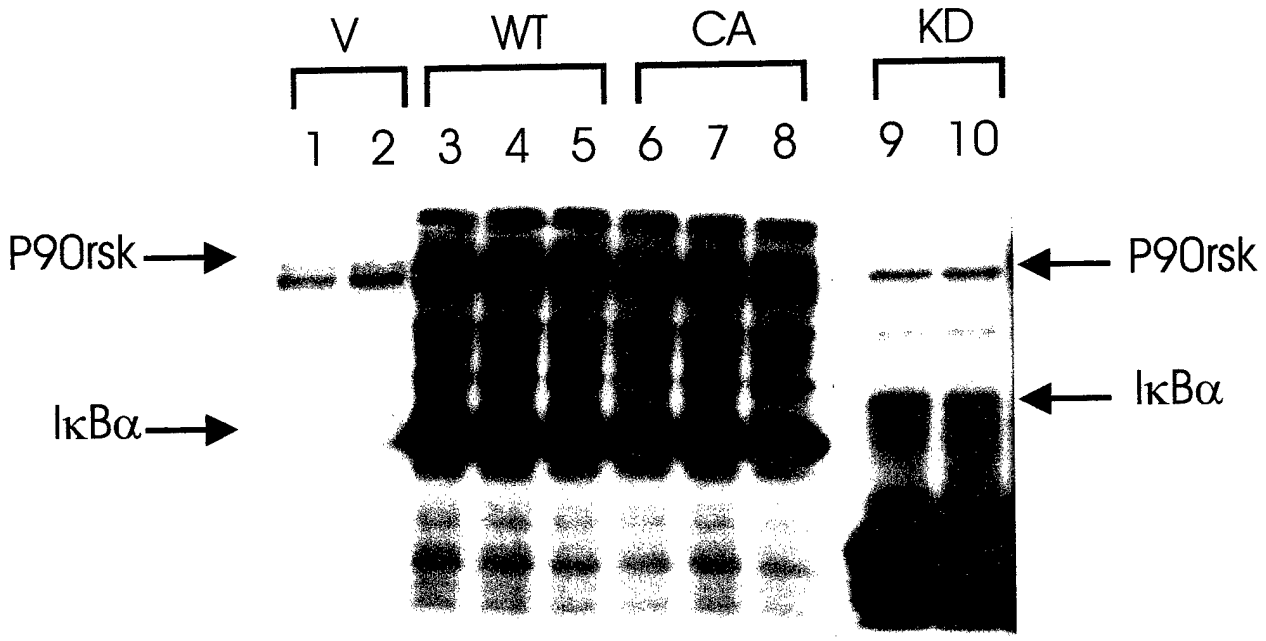

Figure 2: Immunoprecipitation-Linked Protein Kinase Assays of Cell Lines Expressing Alleles of p90rsk. Cells expressing vector (V), wild-type (WT), constitutively active (CA) and kinase-dead (KD) alleles of p90^{rsk} were immunoprecipitated with anti-HA antibody. The extracts analyzed were from: lane 1, HMV-4; 2, MCV-5; 3, HMWT-12; 4, MCWT-13; 5, MCWT-14; 6, HMCA-15; 7, HMCA-16; 8, MCCA-18; 9, HMKD-7; 10, MCKD-9. Lanes 1-8 are films of gels exposed for 1 hour. Lanes 9 & 10 were taken from a separate gel which was exposed for 18 hrs.

precipitable following the precipitation by anti-HA antibody.

Task 5:	Determine the effect of heterologously expressed p90^{rsk} upon the activity of upstream kinases such as ERK and MEK as well as kinases of the PI3-kinase pathway (Akt and p70^{s6k}).
ProposedTime Line:	Year 1/Month 6-7
Actual Time:	To be inititated after Task 6.

We have chosen to look at the problems in Task 6 first before Task 5 because it is a logical extension of work already published by others and is also likely to be more informative if there are no problems in the execution.

Task 6:	Determine the contribution of the PI3-kinase pathway *v.s.* the Ras-Raf-ERK pathway on p90^{rsk1} activation.
Proposed Time Line:	Year 1/ Month 8-12; Year 2: Month 1-4
Actual Time:	In progress

These experiments are presently in progress. Figure 3 depicts a representative experiment performed with two MCF-7 derived cell lines, MCWT-13 and MCCA-18. Both of these cell lines express the transduced p90rsk in a robust fashion (see Figure 1, lanes 13 and 18). Cells were starved overnight for 18 hours in the presence of 1% fetal calf serum (reduced from 10%). The cellls were harvested for the control sample, or treated with 10 ng/ml of IGF-1 or 10 ng/ml of IGF-1 + wortmannin (200 nM). Wortmannin is an inhibitor of PI-3 kinase. Thirty minutes following the addition of growth factor, or growth factor + wortmannin, cells were harvested for IP-kinase assays and bacterially produced IκBα was used as the substrate. The results show that wortmannin appears to inhibit the activation of the wild-type p90rsk but not the constitutively active allele. Future experiments will include a wortmannin only set of samples.

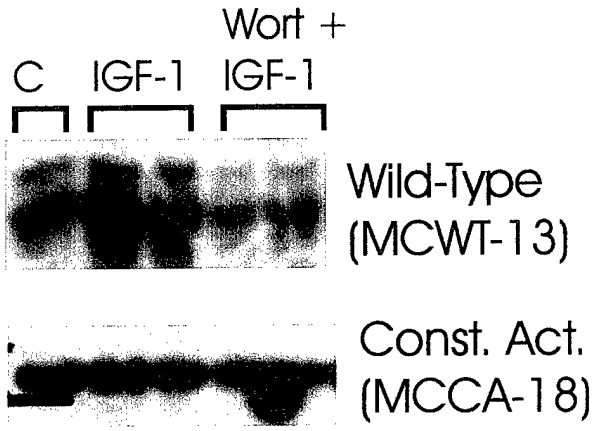

Figure 3: Immunoprecipitation-linked protein kinase activity of MCF-7 cell carrying WT and CA alleles of p90rsk treated with IGF-1 or IGF-1 and wortmannin. Control cells were starved overnight but not treated with either IGF-1 or wortmannin. Cells were either treated with 10 ng/ml of IGF-1 or IGF-1 and 200 nM wortmannin.

KEY RESEARCH ACCOMPLISHMENTS

- The construction and transduction of retroviral constructs of p90^{rsk} into primary breast epithelial cells as well as MCF-7 cells.
- The establishment of cell lines permanently expressing p90^{rsk} alleles.
- Protein and activity expression from transduced genes.
- The inhibition of activation of wild-type p90^{rsk} by wortmannin (to be repeated).

REPORTABLE OUTCOMES

- None at this time

CONCLUSIONS

- P90^{rsk} kinase-dead allele expression is not lethal. This is important because there was a chance that this allele could act efficiently as a dominant-negative gene and suppress the activity of the wild-type gene to the point that the cells could be inviable.

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Ghoda, L., Lin, X., and Greene, W.C.: The 90 kDa Ribosomal S6 Kinase Phosphorylates IκBα and Stimulates Its Degradation *In Vitro. J. Biol. Chem.* **272**: 21281-21288 (1997)

APPENDICES

- None included.